STIMULATION OF DE NOVO SYNTHESIS OF L-PHENYLALANINE AMMONIA-LYASE DURING INDUCTION OF PHYTOALEXIN BIOSYNTHESIS IN CELL SUSPENSION CULTURES OF *PHASEOLUS VULGARIS*

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1. Introduction

L-Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyses the first reaction in the biosynthesis from L-phenylalanine of a wide variety of phenylpropanoid compounds including lignin, coumarins, esters of hydroxycinnamic acids, flavonoids, isoflavonoids and pterocarpans [1]. Fluctuations in PAL levels are thought to be a key element in the control of phenylpropanoid biosynthesis in higher plants [2,3]. Particular attention has been given to the molecular mechanisms regulating PAL levels during light-stimulated accumulation of phenylpropanoids in a variety of systems [4–10], and there is currently intense debate as to whether in seedlings of *Sinapis alba* phytochrome stimulates de novo synthesis of PAL or causes activation of the enzyme from a pre-existing inactive form [11,12].

In contrast, little attention has been given to the regulation of PAL production and removal in relation to the accumulation of phenylpropanoid phytoalexins and other phenolics in infected tissues. Phytoalexins are formed in plant tissues in response to microbial attack, possess antimicrobial activity and are thought to be important factors in plant disease resistance [13]. Accumulation of phaseollin, a pterocarpan phytoalexin of *Phaseolus vulgaris* [14] can be induced by fungal 'elicitor' marcromolecules [15,16] and also by a variety of model inducers including RNase, certain antimetabolites and heavy metal salts [17–19].

Abbreviation: PAL: L-Phenylalanine ammonia-lyase

Induction of phaseollin accumulation is generally accompanied by marked increases in PAL activity [18,20,21]. In the present paper we use the comparative density labelling technique with ^2H from ^2H_2O [22] to demonstrate stimulation of the rate of PAL synthesis de novo in cell suspension cultures of *Phaseolus vulgaris* treated with autoclaved RNase.

2. Materials and methods

Cell suspension cultures of *Phaseolus vulgaris* were grown in a modified Schenk and Hildebrandt medium [23] as described [19]. Cells from log phase cultures were exposed to ^2H_2O by removal of half the culture medium and its replacement with an equal volume of 99.7% ^2H_2O (British Drug Houses Ltd), thereby giving an effective ^2H_2O concentration of 50% (v/v). Parallel experiments were performed ± autoclaved RNase (0.5 mg/ml final concentration, Sigma Chemical Co.). Harvested cells [19] were stored at −173°C until required for PAL assay [23] or CsCl density gradient centrifugation as in [24] using β-galactosidase (EC 3.2.1.23, Boehringer-Mannheim Ltd) from *Escherichia coli* as an external marker enzyme, buoyant density 1.300 kg/l in CsCl [7]. After centrifugation for 40 h at 110 000 g_{av} in an MSE 10 × 10 ml fixed angle (20°) rotor (catalogue no. 2410), 80 four-drop fractions were collected: in each consecutive set of ten fractions, numbers 1,4,7 were each split, 20 μl was assayed for β-galactosidase and the remainder for acid phosphatase (EC 3.1.3.2) [25];

numbers 2,3,5,6,8,9 were assayed for PAL [24]. Buoyant density values were calculated from refractive index measurements on every tenth fraction and corrected according to the position of the external marker β-galactosidase [7].

3. Results and discussion

Following RNase treatment of *Phaseolus vulgaris* cell suspension cultures there is a transient increase in PAL activity followed by accumulation of phaseollin and other isoflavonoid and flavonoid derivatives [19,21]. Growth of cultures on a defined medium lacking exogenous amino acids allows enzyme density labelling with ^2H from ^2H_2O. High concentrations of ^2H_2O are toxic to plant cells [26]. Therefore we have used a final ^2H_2O concentration of 50%: at this concentration there is relatively little perturbation of the level of PAL activity in control cultures, the RNase stimulation of PAL levels is not inhibited (fig.1) and suitable increases in enzyme buoyant density are produced (fig.2,3).

At all stages PAL extracted from RNase treated cultures is heavier than PAL from comparable control

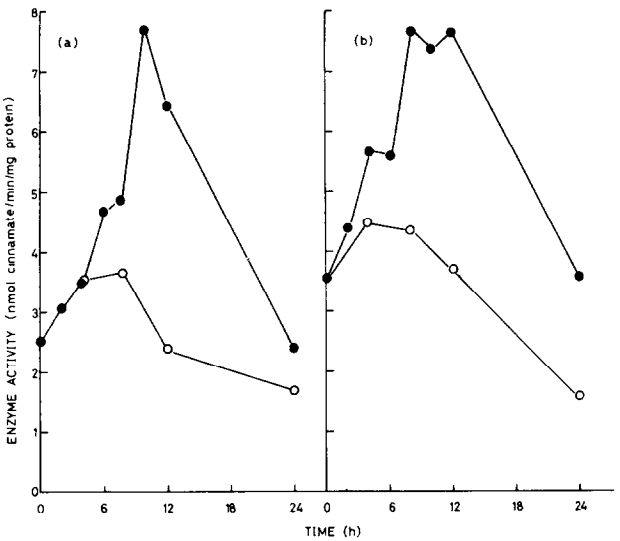

Fig.1. Time-course of the development of PAL activity in RNase treated (–●–) and control (–○–) cultures. At zero time 50% culture medium was removed and replaced with an equal amount of H_2O (a) or 99.7% ^2H_2O (b).

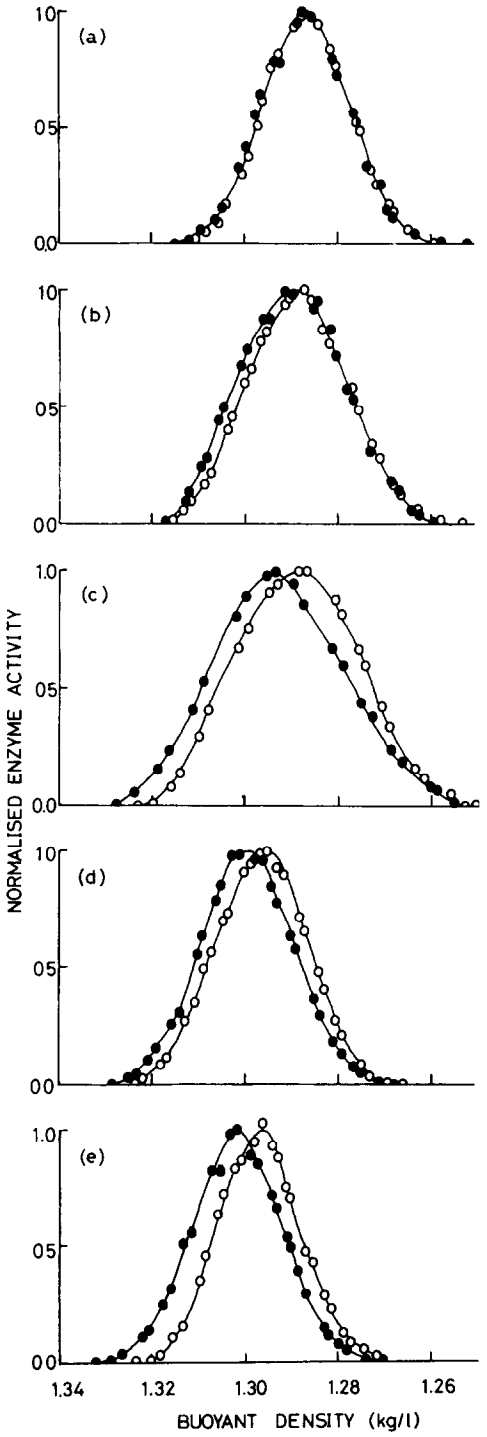

Fig.2. Equilibrium distribution of PAL activity in CsCl density gradients. Enzyme was obtained from RNase treated cultures (–●–) or control cultures (–○–) that had been exposed to H_2O for 10 h (a), or 50% ^2H_2O for 4 h (b), 8 h (c), 12 h (d), 24 h (e).

cultures (fig.2). The experiments fulfil criteria developed for rigorous interpretation of comparative density labelling data [22].

(i) Label and stimulus were applied simultaneously.

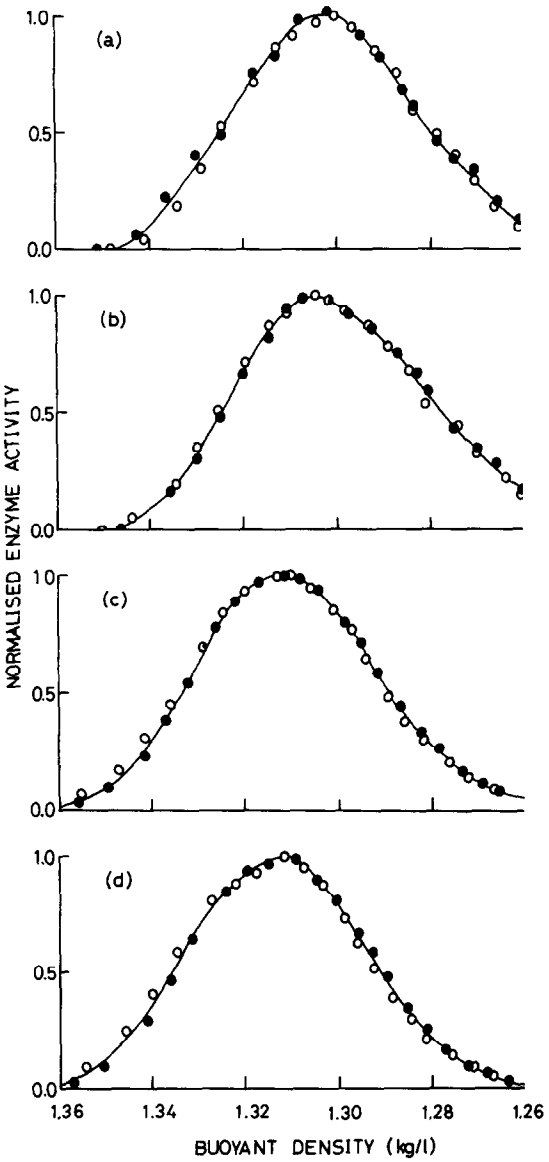

Fig.3. Equilibrium distribution of acid phosphatase activity in CsCl density gradients. Enzyme was obtained from RNase treated cultures (–•–) or control cultures (–○–) that had been exposed to H_2O for 10 h (a), or 50% ^2H_2O for 4 h (b), 12 h (c), 24 h (d).

(ii) Native PAL from RNase treated and control cultures have the same buoyant density.

(iii) Increases in buoyant density of PAL are accompanied by increases in bandwidth implying that the rate of labelling of the enzyme is not limited by the rate of uptake of ^2H into the amino acid pool.

(iv) The enzyme has not completely turned over at time points where comparative measurements are made.

(v) The activity level and rate of labelling (fig.3) of the internal control enzyme acid phosphatase are unaffected by RNase treatment, thereby demonstrating that the specific activity of density label in the amino acid pool is unaffected by the stimulus.

Therefore, the observed differences in the rate of labelling of PAL reflect differences in the turnover of the enzyme and we conclude that RNase treatment leads to an increase in the rate constant for de novo synthesis of PAL. It is worth noting that this rate constant is composite, being for example a function of the rates of transcription, translation and activation of newly synthesised inactive polypeptide precursors [22]. Therefore the results are consistent with but do not yet prove the hypothesis that phytoalexin synthesis is induced by stimulation of transcription of the genes for the appropriate biosynthetic enzymes [27].

The light-mediated stimulation of PAL levels in cell suspension cultures of *Petroselinum hortense* has been shown by radio-labelling and density-labelling methods to involve stimulation of de novo enzyme synthesis [6,28]. Further analysis revealed a transient, light-mediated increase in translatable mRNA for PAL [9]. The demonstration of stimulation of de novo synthesis of PAL accompanying induction of phaseollin accumulation similarly provides a basis for analysis of the molecular mechanisms that regulate phytoalexin production.

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